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ACTION OF RESERPINE IN MORPHINE TOLERANT RATS: ABSENCE OF AN ANTAGONISM OF CATECHOLAMINE DEPLETION

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FOREWORD
(Nontechnical summary)

Previous investigators have demonstrated that animals which have become unresponsive (tolerant) to the analgesic effects of morphine due to repeated doses are also resistant to sedation caused by reserpine. Reserpine is known to exert its sedative effect pharmacologically by interfering with the storage mechanism of catecholamine and indoleamine neurotransmitters located in the synaptic vesicles of nerve cells. As a result of this interference the brain is largely depleted of these neurotransmitters. In morphine tolerant animals, however, most of the catecholamines remain stored after treatment with reserpine. The mechanism of this resistance to reserpine has been investigated to determine if it may provide a clue to the as yet unknown mechanism of morphine tolerance.

The results demonstrate that morphine does not directly alter inhibition of the neurotransmitter storage mechanism by reserpine. Rather, morphine treatment restricts the amount of reserpine which reaches the brain, diminishing the effectiveness of the dose. This was true only for reserpine administered intraperitoneally, as done by previous investigators. Rats treated intravenously were as susceptible as control animals to depletion of catecholamines. It is concluded that reduced effectiveness of reserpine in depleting catecholamines in animals treated chronically with morphine is not related to tolerance by the central nervous system to morphine.

ABSTRACT

The mechanism of resistance to the catecholamine depleting effects of reserpine in morphine tolerant rats was examined. In vitro, morphine did not alter reserpine inhibition of ^{3}H-norepinephrine uptake by synaptic vesicles. Similarly, uptake by vesicles isolated from morphine tolerant animals was as susceptible to reserpine inhibition as that of corresponding preparations from saline treated controls. Consistent with earlier reports, intraperitoneally administered reserpine only partially reduced brain catecholamine levels in most rats treated chronically with morphine. However, reserpine injected intravenously depleted norepinephrine and dopamine in experimental animals to the same extent as controls treated by either route of administration. The amount of reserpine which actually reached the brain was estimated by intraperitoneal injection of ^{3}H-reserpine. Animals chronically treated with morphine and with only partially reduced norepinephrine concentrations exhibited tritium levels less than one-half those of controls. It is concluded that an increased resistance to reserpine cannot be correlated with CNS tolerance to morphine. Rather, degradation or excretion of reserpine may be enhanced peripherally as a result of chronic morphine treatment.

I. INTRODUCTION

Development of tolerance to morphine in rats has been shown to be accompanied by a resistance to the catecholamine depleting effects of reserpine.[7,8] Nearly 60 percent of the catecholamines were shown to remain in whole brains of tolerant animals following an intraperitoneal dose of reserpine which almost completely depleted levels in control animals. Acute treatment with morphine, on the other hand, is reported to only slightly antagonize reduction of amine concentrations by reserpine.[5]

The relative insensitivity to reserpine suggested involvement of the tolerance phenomena in the catecholamine transport and storage mechanisms of synaptic vesicles. Development of tolerance could involve, in part, induced structural and/or functional changes in the synaptic vesicle membrane, which in turn could alter interaction of reserpine with its binding sites. Alternately, competition of the two drugs for the same or interrelated sites could occur as suggested by others.[8]

In the present study, these possible mechanisms of morphine-reserpine interaction have been investigated by measuring the effect of both drugs on ^{3}H-norepinephrine uptake by synaptic vesicles in vitro and by comparing the action of reserpine administered intraperitoneally or intravenously in rats chronically treated with morphine. The data suggest that the relative ineffectiveness of reserpine cannot be ascribed to morphine tolerance in the central nervous system.

II. METHODS

Materials and animals. Male Sprague-Dawley rats (250-300 grams) were used in all experiments. dl-7-^{3}H-norepinephrine (4.23 Ci/mmole) and ^{3}H-reserpine (trimethoxybenzoyl ring labeled, 156.4 mCi/mmole) were purchased from New England

Nuclear Corporation, Boston, Massachusetts. Morphine sulfate was obtained from Eli Lilly and Company, Indianapolis, Indiana (15 mg/ml) for in vivo administration and from Mallinckrodt, St. Louis, Missouri, as a powder for in vitro studies. Reserpine, dopamine and dl-norepinephrine were all purchased from Sigma Chemical Company, St. Louis, Missouri.

Administration of drugs. Morphine was injected intraperitoneally according to three regimens. In acute studies, 60 mg/kg were given, and rats were euthanatized by decapitation 1 hour later. In chronic experiments, morphine was administered twice daily for 7-1/2 days, either with 30 mg/kg or by a step-up schedule starting with 30 mg/kg and increasing the dosage daily by 10 mg/kg to 60 mg/kg. Animals were euthanatized 1 or 6 hours after the last injection.

Reserpine solutions were prepared by dissolving the appropriate quantity of drug in 1 ml of glacial acetic acid and diluting the solution to a final acetic acid concentration of 0.174 M. Reserpine was injected either intraperitoneally (2.5 mg/kg) or via the tail vein (0.5 mg/kg). The latter group was anesthetized lightly with ether prior to administration. In experiments in which radioactive reserpine was injected intraperitoneally, 6.4×10^7 dpm of ^{3}H-reserpine (approximately 30 μCi) were diluted to a final concentration of 1.25 mg/ml with unlabeled reserpine.

In vitro assay for norepinephrine uptake. Synaptic vesicles were isolated by a modification of the methods described by Whittaker et al.[13] and De Robertis and Rodriguez.[2] Following resuspension of the crude synaptosomal pellet in water (2 ml/g original tissue) as described by Whittaker et al.,[13] the homogenate was centrifuged at 20,000 x g for 20 minutes. The supernatant fraction was carefully pipetted off and

further centrifuged at 110,000 x g for 50 minutes in a Beckman Model L2 centrifuge using a 50 rotor. The supernate was discarded and the pellet, containing partially purified synaptic vesicles, was gently resuspended by hand with a glass-Teflon homogenizer in 0.13 M K_2HPO_4 buffer, pH 7.4. As a result of initial experiments, a volume of 5 ml was chosen for resuspension of a pellet obtained from two pooled rat brains (approximately 3.5 g of tissue).

Uptake of norepinephrine was measured by a modification of the assay described by Euler and Lishajko.[4] The resuspended vesicles were preincubated at 23°C for 40 minutes, and 0.5-ml aliquots (equivalent to approximately 0.35 g of brain) were added to 0.5 ml of a reaction mixture. The 1-ml assay volume at pH 7.4 contained in final concentration 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.13 M K_2HPO_4, and 6×10^{-7} M ^{3}H-dl-norepinephrine (340,000 dpm). Tubes containing the assay mix were immediately placed in a water bath shaker at 23°C. Uptake of ^{3}H-norepinephrine was stopped by addition of 7 ml of ice-cold dl-norepinephrine solution (6×10^{-5} M). Samples were centrifuged at 110,000 x g for 45 minutes, supernatant fractions discarded, and the pellets washed once with 2 to 3 ml of water. The pellet was resuspended in 1 ml of water and counted in 10 ml of Aquasol (New England Nuclear) in a Nuclear-Chicago Mark II scintillation counter (efficiency of 45 percent). Blanks were prepared by mixing the reaction mix and vesicle suspension at 0°C, followed immediately by 7 ml of the cold norepinephrine solution. These blanks were indistinguishable from samples incubated at 0°C for several minutes.

Quantitation of catecholamines and ^{3}H-reserpine. Catecholamines were extracted according to the method described by Weil-Malherbe.[12] Norepinephrine and dopamine

were determined by known fluorometric assays.[10,12] The efficiency of catecholamine recovery from alumina was estimated either by adding known amounts of ^{3}H-norepinephrine to brain homogenates and determining the radioactivity in the acetic acid eluate from the column, or by treating a known amount of norepinephrine solution according to the alumina extraction procedure and quantitating the recovered amine by fluorometric assay. Recoveries were 77 ± 5 percent by either procedure.

^{3}H-reserpine was extracted and radioactivity determined according to the method of Alpers and Shore.[1] In these studies, brains previously frozen in liquid nitrogen and stored at -70°C were powdered at Dry Ice temperature and duplicate 0.3-g quantities were extracted for reserpine. The efficiency of the extraction procedure was estimated by adding known amounts of ^{3}H-reserpine to brain homogenates. Recoveries were approximately 80 percent.

Assessment of tolerance. The hot plate method of Eddy and Leimbach,[3] as modified by Jóhannesson and Woods,[9] was used to measure tolerance. Rats were placed on a hot plate at 55 ± 0.5°C within a Plexiglas restraining cylinder. The time interval between contact with the plate and reaction to the stimulus (licking of paws or jumping) was used as an index of analgesia, unresponsive animals being arbitrarily removed after 60 seconds. Rats were tested prior to administration of morphine or saline and again 1 hour after the first injection. One-half hour after the last injection of the tolerance regimen, control and experimental animals were tested a third time. Rats with reaction times less than 25 seconds following the initial injection of morphine were discarded.

Statistics. The data were analyzed by the Student's "t" test.

III. RESULTS

Uptake of ^{3}H-norepinephrine by synaptic vesicles in vitro. Initial experiments were made to determine linearity and energy requirements of the transport system. Uptake of ^{3}H-norepinephrine (6×10^{-7} M) was linear for 3 minutes, the rate decreasing sharply thereafter (Figure 1A). Doubling the ATP concentrations from 2.5 to 5 mM failed to increase the initial rate of accumulation (Figure 1A). However, the rate of uptake remained almost linear after 5 minutes, suggesting that loss of ATP can become a limiting factor on transport rate at longer incubation times. In the absence of added

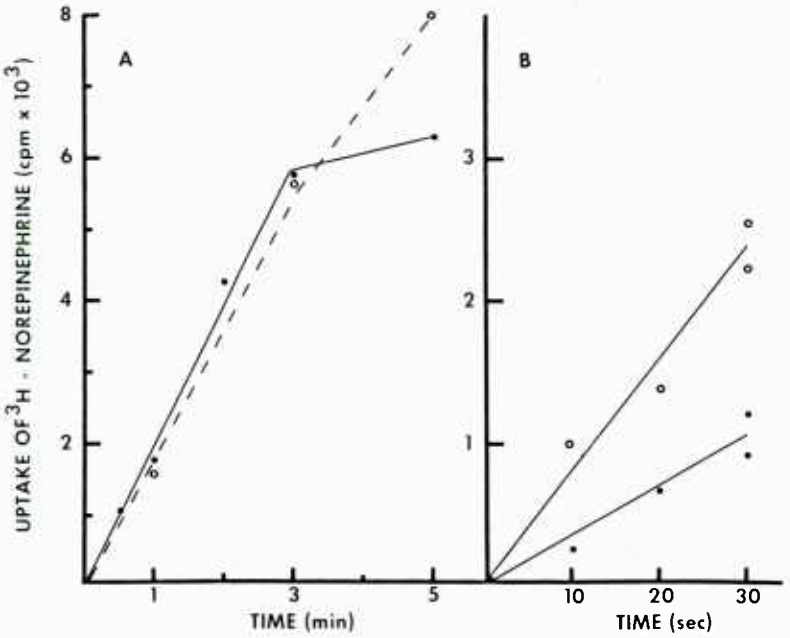

Figure 1. Uptake of ^{3}H-norepinephrine by synaptic vesicles.
A. Uptake is expressed as cpm/0.5 ml vesicle suspension. Values are corrected by blanks. ●—● 2.5 mM ATP o---o 5.0 mM ATP
B. Uptake is expressed as cpm/quantity of vesicle suspension added. o—o 1.0 ml vesicle suspension ●—● 0.5 ml vesicle suspension

ATP and Mg^{++} there was a slower rate of tritium accumulation which essentially stopped after 1 minute (not shown). This accumulation could be completely abolished with either 3 mM EDTA or 10^{-6} M reserpine.

The rate of ^{3}H-norepinephrine uptake was proportional to synaptic vesicle content (Figure 1B). Doubling the quantity of vesicle suspension added to the assay increased the uptake of tritium from 2100 cpm/min to 4700 cpm/min.

Effect of morphine and reserpine on ^{3}H-norepinephrine uptake by synaptic vesicles. Morphine alone appeared to have little effect on norepinephrine transport in vitro (Table I). Vesicles isolated from either chronically or acutely morphine treated rats exhibited uptake rates similar to those of control animals. Likewise, 10 or 100 μM morphine added in vitro had no significant effect on uptake.

To test the possibility of morphine-induced desensitization of synaptic vesicle transport to reserpine, ^{3}H-norepinephrine uptake was measured in the presence of varying concentrations of reserpine (Table II). Vesicles from morphine tolerant animals appeared as susceptible to reserpine inhibition as those from controls. In the presence of both reserpine and morphine, vesicles from control rats showed an inhibition of uptake comparable to that of reserpine added alone (Table II).

Effect of reserpine in vivo on brain catecholamine levels. Reserpine was administered in vivo to determine if the reported protection to reserpine afforded by chronic morphine treatment could be confirmed. To avoid masking a protective mechanism by using an overwhelming dose of reserpine, the minimum quantity of drug (2.5 mg/kg i.p. or 0.5 mg/kg i.v.) needed to deplete at least 80-90 percent of catecholamine stores in

Table I. Effect of Morphine on ^{3}H-Norepinephrine Uptake In Vitro by Synaptic Vesicles*

Regimen of treatment with morphine	Uptake of tritium (percent)
Acute (60 mg/kg)	90 ± 11 (4)
Chronic (30 mg/kg)	102 ± 2 (3)
Control + 10 μM morphine in vitro	94 ± 6.5 (4)
Control + 100 μM morphine in vitro	88.5 ± 18.5 (5)

* Pairs of animals were euthanatized 1 hour after the last injection of morphine or saline and uptake of tritium by isolated synaptic vesicles was determined at various time periods up to 1 minute. Rates were calculated as cpm/min per 0.5 ml vesicle suspension and are expressed as percent of the uptake rate of vesicles isolated from a corresponding pair of control animals euthanatized the same day. In vitro additions were made to a portion of a vesicle suspension from control animals 1/2 hour prior to the assay. The remaining untreated suspension served as a control. Numbers in parentheses refer to the number of experiments. Values are expressed ± s.d.

Table II. Effect of Reserpine In Vitro on ^{3}H-Norepinephrine Uptake by Synaptic Vesicles from Chronically Morphine Treated or Control Rats*

Additions to assay	Control (percent)	Morphine tolerant (percent)
10^{-8} M reserpine	41 ± 3.3 (4)	47 ± 6.4 (4)
10^{-7} M reserpine	19 ± 9.0 (4)	16 ± 8.3 (4)
10^{-6} M reserpine	9 ± 4.6 (4)	7.5 ± 5.2 (4)
5×10^{-9} M reserpine	86	--
5×10^{-9} M reserpine + 100 μM morphine	88 ± 3 (2)	--
10^{-7} M reserpine + 100 μM morphine	25 ± 2 (2)	--

* Uptake of tritium by synaptic vesicles isolated from control and chronically morphine treated (30 mg/kg) animals was measured over a 1-minute period. Reserpine and morphine were added to 0.5 ml of vesicle suspension 15 minutes and 30 minutes, respectively, prior to addition of reaction mix. Uptake is expressed as percent of uptake in the absence of any additions. Numbers in parentheses refer to number of experiments (one control and one experimental animal per experiment). Values are expressed ± s.d.

controls was estimated from a dose response curve. Rats made tolerant to 60 mg/kg morphine were given reserpine intraperitoneally 2 hours after the last morphine injection and euthanatized 4 hours later. As shown in Table III, levels of norepinephrine and dopamine were only partially diminished in the majority of morphine tolerant animals given reserpine intraperitoneally. The relatively normal behavior of these animals was in sharp contrast with the sedation of both controls and those morphine treated animals in which catecholamines were largely depleted.

The development of two populations (reserpine-resistant and reserpine-sensitive) within the experimental group following intraperitoneal reserpine administration could not be explained by a variation in degree of tolerance as determined by the hot plate test. Reaction times were 11 $\pm$ 5 seconds for morphine tolerant, reserpine-resistant; 13 $\pm$ 8 seconds for morphine tolerant, reserpine-sensitive; and 5.5 $\pm$ 2.2 seconds for the control.

The resistance to catecholamine depletion in morphine tolerant rats did not occur when reserpine was administered intravenously. Catecholamine levels in all experimental animals were not statistically different from those in either similarly treated or intraperitoneally injected controls (Table III).

Levels of ^{3}H-reserpine in the brain. To test the possibility that less intraperitoneally administered reserpine was reaching the brain in rats chronically treated with morphine, levels of ^{3}H-reserpine were measured in brains of control and experimental animals following intraperitoneal injection. As seen in Table IV, reserpine levels in morphine tolerant animals with only partially reduced norepinephrine concentrations were less than half those found in controls.

Table III. Effect of Reserpine In Vivo on Levels of Catecholamines in Control and Chronically Morphine Treated Rats*

Reserpine	Control	Morphine tolerant
	Norepinephrine (ng/g tissue)	
0	498.5 ± 66.7 (4)	417.3 ± 71.9 (4)
2.5 mg/kg i.p.	82.1 ± 20.9 (5)	360.8 ± 67.1† (8)
		72.2 ± 6.6 (4)
0.5 mg/kg i.v.	89.9 ± 11.5 (6)	67.2 ± 9.9 (6)
	Dopamine (ng/g tissue)	
0	1002 ± 155 (4)	965 ± 47 (4)
2.5 mg/kg i.p.	68.6 ± 29.8 (4)	731.2 ± 78.5† (4)
		72.1 ± 8.4 (2)
0.5 mg/kg i.v.	119.6 ± 29.3 (6)	77.2 ± 15.3 (6)

* Two hours after the last morphine (60 mg/kg) or saline injection, reserpine (2.5 mg/kg i.p. or 0.5 mg/kg i.v.) was administered. Animals were euthanatized 4 hours later. Numbers in parentheses refer to number of animals. Catecholamine levels are expressed ± s.d.

† $P < 0.001$; degree of significance, morphine treated compared with control

Table IV. Levels of Reserpine and Norepinephrine in Whole Brain of Control and Chronically Morphine Treated Rats*

	Reserpine (ng/g tissue)	Norepinephrine (ng/g tissue)
Control (5)	15.8 ± 1.3	82.1 ± 20.9
Morphine tolerant (4)	6.8 ± 1.0†	325.6 ± 40.3†
Morphine tolerant (2)	15.8 ± 1.6	68.4 ± 4.2

* Two hours after the last saline or morphine injection (60 mg/kg) ^{3}H-reserpine was administered intraperitoneally (2.5 mg/kg, 60 μCi/2.5 mg reserpine). Animals were euthanatized 4 hours later. Values are expressed ± s.d. Numbers in parentheses refer to number of animals.

† $P < 0.001$; degree of significance, morphine treated compared with control

IV. DISCUSSION

The results of this study strongly suggest that CNS tolerance to morphine is not involved in the observed resistance of amine stores to depletion by reserpine administered intraperitoneally. In earlier studies longer treatment regimens utilizing higher doses of morphine were used to elicit this effect.[7,8] However, reserpine was given intraperitoneally in every case. In our studies, most of the animals exhibiting tolerance to morphine also demonstrated a large degree of insensitivity to reserpine-induced depletion of amines provided the reserpine was administered intraperitoneally (Table III). This phenomenon could be abolished by intravenous administration of the drug at a dose which was essentially equivalent (in terms of reduction in catecholamine levels) to the quantity given intraperitoneally (Table III). This suggests that reserpine injected intraperitoneally may be preferentially metabolized or absorbed peripherally as a result of chronic morphine treatment, thus reducing its effectiveness in depleting brain catecholamines. That less reserpine reached the brain in tolerant animals, as compared to controls, was supported by the observation that lower levels of radioactivity were present in the brain following injection of ^{3}H-reserpine.

A small number of tolerant animals were found to be sensitive to intraperitoneally administered reserpine. This could imply that in these animals an elimination mechanism of intraperitoneally injected reserpine may only be marginally induced by our tolerance schedules. Studies[7,8] which utilized larger doses and a more prolonged treatment showed all animals to be largely resistant to amine depletion by reserpine.

The in vitro assay permitted direct measurement of the effect of reserpine and morphine on norepinephrine transport by synaptic vesicles. The mechanism of

depletion of catecholamine stores by reserpine involves inhibition of uptake and storage as demonstrated *in vivo*[6] as well as *in vitro* with adrenergic nerve granules[4] and hypothalamic vesicles.[11] Results of our studies are consistent with our *in vivo* findings. There was both a lack of a direct protective effect by morphine toward reserpine inhibition as well as an unchanged sensitivity of the transport system toward reserpine after development of tolerance (Table II).

Based on these findings, it is concluded that the acquired resistance to the amine depleting effects of intraperitoneally administered reserpine is not involved in the phenomena of CNS tolerance to morphine.

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13. ABSTRACT

The mechanism of resistance to the catecholamine depleting effects of reserpine in morphine tolerant rats was examined. In vitro, morphine did not alter reserpine inhibition of ^{3}H-norepinephrine uptake by synaptic vesicles. Similarly, uptake by vesicles isolated from morphine tolerant animals was as susceptible to reserpine inhibition as that of corresponding preparations from saline treated controls. Consistent with earlier reports, intraperitoneally administered reserpine only partially reduced brain catecholamine levels in most rats treated chronically with morphine. However, reserpine injected intravenously depleted norepinephrine and dopamine in experimental animals to the same extent as controls treated by either route of administration. The amount of reserpine which actually reached the brain was estimated by intraperitoneal injection of ^{3}H-reserpine. Animals chronically treated with morphine and with only partially reduced norepinephrine concentrations exhibited tritium levels less than one-half those of controls. It is concluded that an increased resistance to reserpine cannot be correlated with CNS tolerance to morphine. Rather, degradation or excretion of reserpine may be enhanced peripherally as a result of chronic morphine treatment.

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